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Evaluation of the nucleolar organizer region associated proteins in exfoliative cytology of normal buccal mucosa. Effect of smoking

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Abstract

An evaluation of cellular alterations in the smoker's oral mucosal cells was performed. The Exfoliative Cytology technique was applied and the cytological smears stained with silver for the enumeration of Argyrophilic nucleolar organizer regions (AgNORs). Cytological smears were collected from two anatomic sites: floor of the mouth and tongue border, in both groups, smokers and non smokers, with a purpose of correlating the smoking habit to possible cellular alterations. The enumeration of the AgNORs showed that the average number of AgNORs is higher in smokers. There is a significant difference ($P=0.0001$) between smears from the floor of the mouth and from tongue border in the smoking group. In this study, no correlation between number of cigarettes, age and gender was found, but the results suggest that there might be a correlation between the smoking habit and an increased rate of cellular proliferation in the oral mucosal cells. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Cytology; Argyrophilic nucleolar organizer regions; Smoking; Oral mucosa; Oral neoplasm; Prevention

1. Introduction

Cancer has been considered a public health problem all over the world, as this disease affects at least 9 million and kills about 5 million people every year, being currently the second cause of death in most of the countries (not considering the external causes of death). The incidence of cancer tends to raise about 100% within the next 20 years, if prevention and control measures are not taken (World Health Organization) [57].

The efforts made towards cancer prevention are based on monitoring the changes of risk factors exposure that are related to the tumor development, being traditionally done by health education, in individual or group basis, through media or other sources [1].

In spite of these efforts and the fact that the mouth is an easily accessed region for the examination, oral cancer diagnosis is still late and about half of the diagnosed patients die of the disease [2,3].

Exfoliative cytology is a diagnosis complementary exam, based on the observation of cells that are constantly exfoliating from the epithelium, as a consequence of this tissue's natural maturation process [4,5]. Many studies have suggested the use of exfoliative cytology in an oral cancer prevention program, as an auxiliary resource to early diagnosis of these lesions [6–14].

According to Girod et al. [15], if genetic changes are accumulated during carcinogenesis and precede the malignant transformation, markers of these molecular or cellular changes should be identified in specific stages of the disease.

Nucleolar organizer regions (NORs) are ribosomic DNA loops and associated proteins that run on the fibrillary centers and the dense fibrillary components of the cell nucleolus, during the interphase, and are responsible for the ribosomic RNA copy.

The silver soaking method was developed by Howell et al. [16], with the purpose of staining the NORs in a differentiated way [17]. In 1982, Ploton et al. [18] adapted the silver soaking technique to observe the Argyrophilic

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NORs (AgNORs) into a one-step technique, which permitted them to observe these structures under the optical microscope, and in 1983 concluded that the silver stained proteins were basically formed by RNA polymerase I, C_{23} and B_{23}, directly involved in nucleolus DNA and ribosomic RNA copy. The use of the AgNORs silver soaking technique in exfoliative cytology finds support in the works done by Sujathan et al. [19], Metze and Lorand-Metze [20], Cardillo [21] and Sampao et al. [22].

Sampao et al. [22] used this technique in exfoliative cytology smears collected from the clinically healthy oral mucosal cells, comparing smokers and non-smokers. These authors showed that the number of AgNORs, as well as the frequency of nucleuses with more than five AgNORs is higher within the smokers group, indicating an increase in the proliferative activity of healthy oral mucosal cells in smokers. However, additional studies are needed to evaluate the importance of these findings in the oral cancer development.

This study, which aims at contributing to the search for methods of early diagnosis of oral cancer that might facilitate monitoring, diagnosis and consequent life improvement of the patients, proposes to evaluate through the count of AgNORs, the use of the AgNORs silver soaking technique on clinically healthy oral mucosal cell smears, obtained through exfoliative cytology technique, in patients over 50 years old, filtered cigarette smokers.

The following purposes were set: (1) calculate the average number of AgNORs and the average percentage of nucleuses with more than three AgNORs observed in the clinically healthy oral mucosal cells of smokers between 50 and 70 years old; (2) compare the average number of AgNORs and the average percentage of nucleuses with more than three AgNORs observed in these cells, to those found in non-smokers of the same age; (3) compare the average number of AgNORs and the average percentage of cells with more than three AgNORs present in the cells from the tongue border and from the floor of the mouth in both groups regardless of being smokers or non-smokers; (4) correlate age and gender of smokers to the amount of AgNORs per nucleus; and (5) correlate the smoking habit frequency to the amount of AgNORs per nucleus.

2. Methods and materials

The population examined in this study comprises individuals who undergo treatment in the "Serviço de Atendimento de Urgência Odontológica do Hospital Nossa Senhora da Conceição" (HNSC) in Porto Alegre, Rio Grande do Sul, Brazil.

One-hundred and twenty individuals among 3304 individuals attending the hospital between March and July 1999 were initially selected. They were selected by order of attendance, that is, in a consecutive way, through preliminary anamnesis.

The sample for this study comprises individuals: (1) between 50 and 70 years old; (2) free from any systemic diseases; (3) non-alcoholic; and (4) with no early or present history of benign or malignant neoplasias.

Two groups were separated: smokers and non-smokers, being considered smokers individuals that smoked over 20 cigarettes/day [23] or 10 cigarettes/day over 10 years.

Only smokers of filtered cigarettes were included in this research. Pipe smokers or consumers of tobacco in other ways were not included, because of concentration variation, which may affect the oral mucosal cells with different intensity, and other systemic effects [24]. Non-smokers were defined as people who had never smoked before. Therefore, former smokers were not included in this research. An elaborate physical intra-mouth examination was performed, and people with any sort of mucosal alterations were discarded.

Groups comprised 60 smokers (20 females and 40 males) and 60 non-smokers (38 females and 22 males).

Collects were performed in both groups, through exfoliative technique, from two anatomic sites: tongue border and floor of the mouth. First the patient had to rinse his mouth with saline solution at 0.9%, and then the collect was performed using a wooden pallet. The material obtained was smeared over a microscopic slide, which was identified by the patient's clinical chart number.

Four microscopic slides were obtained from each patient, two from the tongue border and two from the floor of the mouth. The slides were stored in Borel type jars, filled with ethanol at 95% as fixing agent.

Two of the four slides obtained from each patient were stained using the Papanicolaou Method [25], and the other two using the silver soaking technique, which was adapted by Ploton et al. [26] with an incubation period of 20 min at 45°C. One slide from the tongue border and one from the floor of the mouth was used for each technique performed.

The Papanicolaou's method stained microscopic slides were read in all of its extension, and classified according to the parameters defined by Papanicolaou and Traut [27].

The silver soaked smears were horizontally analyzed in all of its extension, from left to right. The nucleuses from the first 100 cells observed were included in the counting. Cells with no nucleus were not included.

The parameters used on the counting were defined by Crocker et al. [28], that is, the black dots inside the well-defined nucleuses were counted, being the black aggregates (overlapped or merged black dots) counted as one structure. The first 20 slides were counted in a non-consecutive way three times, to calibrate the observer.

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It was observed for each group in each anatomic site: (1) the average number of AgNORs per nucleus; and (2) the average percentage of nucleuses with more than three AgNORs. The data analysis was performed after having the counting of AgNOR's, the information of the clinical charts and the classification of the Papanicolaou modified method stained smears. The sample was characterized, first, setting the variables, which were: age, gender and the classification of the Papanicolaou method stained smears (expressed by the average±standard deviation). The agreement rate among the observers related to the silver soaked smears reading for the AgNOR's counting, was first calculated through the Friedman's non-parametric test.

The average number of AgNORs obtained per slide and the average percentage of nuclei with more than three AgNORs were compared in both groups, smokers and non-smokers, for each anatomic site (tongue border and floor of the mouth); and between the two anatomic sites within the same group, through the Student *t*-test (bi-caudal) for independent samples.

Age, gender, number of cigarettes/day and time (years) of smoking of this sample were correlated to the results found by the analysis of AgNORs, through correlation analysis, calculating the Pearson correlating coefficient.

3. Results

This sample comprised 120 individuals divided into two groups: smoking and non-smoking in a total of 60 individuals in each group. The non-smoking group comprised 22 male individuals (36.66%) and 38 female individuals (63.33%). Of these, 30 individuals (50%) were between 50 and 60 years old and 30 individuals (50%) between 61 and 70. The smoking group comprised 40 male individuals (66.66%) and 20 female individuals (33.33%), being 43 individuals (71.66%) between 50 and 60 years old and 17 individuals (28.33%) between 61 and 70 years old.

The results obtained through the analysis of the slides stained by Papanicolaou's Method were as follows. In non-smoking individuals, 32 tongue border smears were classified as class I and 28 as class II of Papanicolaou, corresponding to 53.33% and 46.67%, respectively, of the 60 smears carried out with the smoking group of this area. The same results: 32 smears class I (53.33%) and 28 class II (46.66%) were obtained in the classification of the slides corresponding to the material collected from the floor of the mouth of the same individuals. In smoking individuals the smears were classified as class I in 38 slides (63.33%) and class II in 22 (36.37%) when considering the slides that corresponded to the material collected from the tongue border. The slides obtained from the floor of the mouth in this group were classified as class I in 41 smears (51.67%) and as class II in 29 (48.33%).

A counting of the AgNORs in the slides soaked by silver through the AgNOR technique adapted by PLOTON was carried out. Initially, the 20 first slides were counted in a non-consecutive way for three times. The analysis of the data obtained from this reading was submitted to the Friedman non-parametric test, where it was found there was a concordance between the readings performed.

The total average of AgNORs per nucleus varied in the sample between 2.39 and 1.3 AgNORs/nucleus. In the smokers group the number of AgNORs per nucleus varied between 2.39 and 1.72 AgNOR/nucleus with an average of 1.94±0.13 AgNORs/nucleus in the smears collected from the tongue border and of 2.07±0.19 AgNORs/nucleus in those collected from the floor of the mouth. In the non-smokers group the average of AgNORs per nucleus varied between 2.2 and 1.3, with an average of 1.79±0.21 AgNORs/nucleus found in the smears from the tongue border and of 1.84±0.23 Nucleus/nucleus in those smears from the floor of the mouth.

A significant variation in the average of AgNORs/nucleus was observed among the smokers, depending on the anatomic site investigated. A higher average was found in the AgNORs/nucleus in the mouth floor ($P=0.0001$). Among the non-smoking patients there was no significant differences in the average of AgNORs/nucleus between the two anatomic sites considered. When comparing groups of the same anatomic site, a significant difference was observed in the average of AgNORs/nucleus between smokers and non-smokers both in the tongue border and the floor of the mouth, with higher numbers in the smoking than in the non-smoking group ($P=0.0001$; Fig. 1).

The percentage of nucleus with more than three AgNORs varied in the smoking group from 14 to 53% and in the non-smoking from 4 to 20%. The number of AgNORs per nucleus observed were calculated by the average percentage of nucleuses with more than three AgNORs was of 25.16±6.01% on the tongue border of the smoking group, while on the floor of the mouth an average of 30.51±8.25% was found. Among the non-smoking individuals, on the tongue border smears, the average percentage of nucleuses with more than three AgNORs was of 11.85±4.50%, while on the floor of the mouth smears an average of 12.80±4.58% was found (Fig. 2).

The results from the tongue border smears with the ones from the floor of the mouth among the smokers group, showed a significant difference on the average percentage of nucleuses with more than three AgNORs in these two anatomic sites was found ($P=0.0001$), being the average higher on the floor of the mouth smears. That was not true for the non-smokers group,

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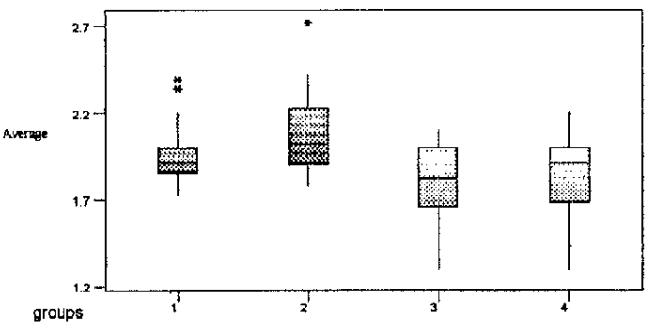

Fig. 1. Box plots showing the distribution of the average number of AgNORs per nucleous according to the groug and anatomic site. (Source: Population examined in the HNSC, Porto Alegre, RS, Brazil, 1999 [65].)

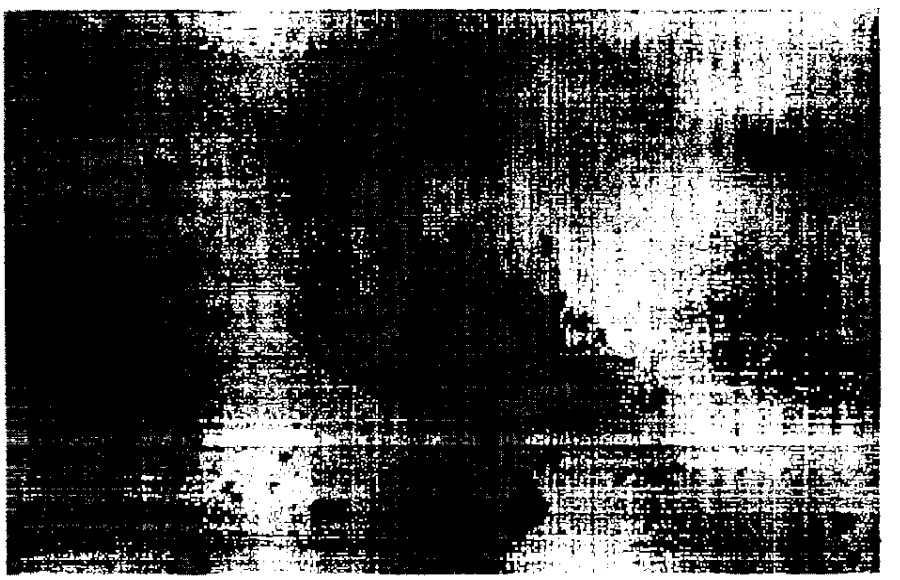

Fig. 2. AgNORS in cells of normal oral mucosa of non smokers (AgNOR stain 1000×).

where the differences between the two anatomic sites were not significant (Fig. 3).

An evaluation performed in the smokers and non-smokers group, on the same anatomic site, showed significant differences on the average percentage of nucleuses with more than three AgNORs on both anatomic sites, being the values higher in the smokers group than in the non-smokers group ($P = 0.0001$; Fig. 4).

The percentage of nuclei with more than five AgNORs varied from 0 to 6% in the sample, varying from 0 to 6% in the smokers group and 0 to 2% in the non-smokers group. The average percentage of nucleuses with more than five AgNORs in tongue border smears from the smokers group was of 1.05%, and of 1.65% for the smears from the floor of the mouth. In the non-smokers group, the average was of 0.27% for both anatomic sites.

It was not possible to correlate age, gender, number of cigarettes smoked a day and the smoking habit time, to the average of AgNORs per nucleus or the percentage of nuclei with more than three AgNORs found in this sample.

4. Discussion

There is a need for studies that point to the direction to be adopted in oral cancer prevention and control measures and to the development of strategies that increase the population participation [29]. Monitoring the appearance of lesions in specific groups that may be classified as risk groups is an effective method of preventing cancer [30,31]. Exfoliative cytology is an adjunct method of monitoring these risk groups, in the long-term

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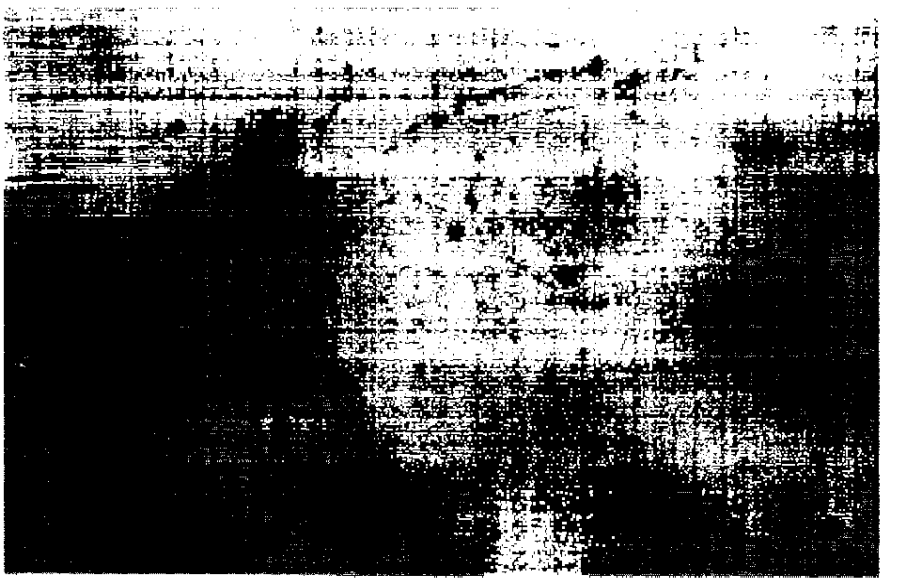

Fig. 3. AgNORS in cells of normal oral mucosa of smokers (AgNOR stain 1000×).

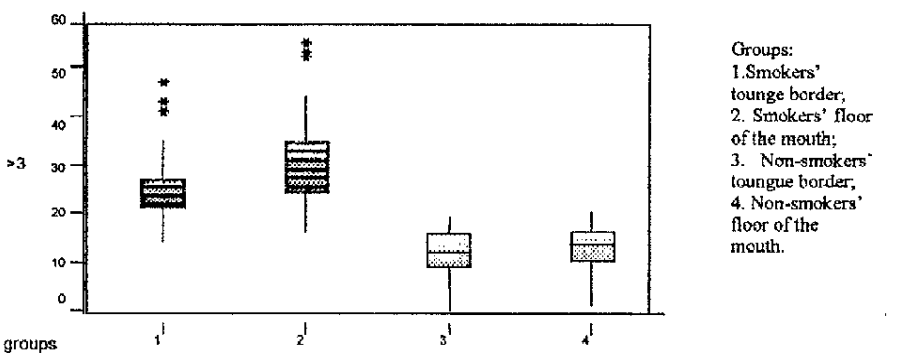

Fig. 4. Box plots showing the distribution of the average of nucleous with more than 3 AgNORs per person according to the group and anatomic site. (Source: Population examined in the HNSC. Porto Alegre. RS. Brazil. 1999 [65].)

control of appearing of oral mucosal epithelium alterations. The effectiveness of the use of quantitative techniques and markers in cytology, like DNA analysis, cytokeratin expression analysis and the use of markers like PCNA and Ki67, has been related by many authors, including Bongers et al. [32] and Ogden et al. [33,34].

In this study the silver soaking technique was used for a evaluation of AgNORs in smears from smokers and non-smokers patients, since this is an effective method to determine ploidy and evaluate the epithelium cells proliferative activity, as related by Hernandez-Verdum et al. [35], Kacerovska et al. [36], Ploton et al. [37,38], Cabrini and Schwint [39] and Van Diest et al. [40].

In the smears obtained for this study, we observed that the nucleolus, as well as the AgNORs, which were visualized as well-defined black dots inside the nucleolus, were well-defined. Such findings support the effectiveness of this method when applied in cytological material, as mentioned by Morgan et al. [41], Cardillo [21], Sujathan et al. [19], Metze and Lorande-Metze [20] and Sampio et al. [22].

Sujathan et al. [19] suggest that by using smears, the study of AgNORs is more precise, since the entire nucleolus can be analyzed, and not only part of it as occurs in histological analyzed tissue cuts. This affirmative is also supported by Crocker et al. [10] and Sampaio et al. [22].

Even though the smears obtained for this research presented enough quantity and quality for the counting, it was observed that the obtaining of a clear field was made difficult by what is believed to be a silver affinity to mucous, food residues and other debris, frequently

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present in oral cavity smears obtained through the exfoliative cytology technique. In the same way, the use of commercial alcohol, which does not pass through an elaborate purity degree control, may have influenced the quality of the microscopic slides obtained.

Cells with no nucleus and no AgNORs were present but not included in the analysis. Nuclei presenting overlapped or merged AgNORs (aggregates) were observed, as related by Crocker et al. [28], Giri et al. [42] and Cabrini and Schwint [39] as well as a pattern variation in the AgNORs distribution by the nucleus.

It seems that the number and the size of the AgNORs establish an inverse standard, being bigger and in smaller number than in a normal epithelium [19,21,28,39, 43,44]. The variations noticed in the distribution pattern of the AgNORs in this study suggest that this is a real tendency. A variation in the size and shape of the AgNORs related to its number was observed. Further cytometric and qualitative studies and a comparative evaluation of the results of these with the effects of the cigarettes on the oral mucosa would be necessary to confirm these observations.

From the affirmatives made about the usefulness of the counting of the AgNORs to establish the ploidy cellular and the proliferative standard of the epithelium [35,36,38–40,42,45,46] and based in the evaluation criteria suggested by Giri et al. [42] and Mourad et al. [46], we observed that the variation in the number of AgNORs per nucleus in the smokers and non-smokers group (2.39 to 1.3 AgNORs/nucleus) in this study defines these cells as diploids and the variation of the percentage of nuclei with more than five AgNORs between 0 and 6% suggests a proliferative pattern of the epithelium that varies in intensity, but remains within a benignity pattern.

Significant differences in the counting of the number of AgNORs per nucleus among smokers and non-smokers were observed in our studies ($P = 0.0001$) suggesting that the smoking may cause alterations in the genetic patterns of the cellular regulation, findings that corroborate those found by Sampaio et al. [22], where an average of 3.4±0.54 AgNOR per nucleus in patient smokers and of 2.6±0.49 AgNORs/nucleus in patients non-smokers. These values differ considerably from those found in our study for smokers in tongue border and floor of the mouth smears (1.94±0.13 and 2.07± 0.19, respectively) and in non-smoking individuals, from the same sites (1.79±0.21 and 1.84±0.23)

The silver soaking technique varied only in the time adapted for the use in cytological material: 30 min in the study of Sampaio et al. [22] and 20 min in this study. There is a need for standardizing the silver soaking technique used in smears obtained through exfoliative cytology technique in oral mucosa, but it does not seem that this time variation explains the discrepancies found in the results of this study.

Consistent differences found in the present study and in that carried out by Sampaio et al. [22] lie in the characteristics of the sample used. In their study, Sampaio et al. [22] examined 40 individuals divided into two groups of 20 people, smoking and non-smoking. The present study comprised 120 individuals, divided into two groups of 60. The larger number of the sample tends to minimize the over-valuation of extreme variations in the number of AgNORs/nucleus, approaching the data obtained in the real average.

Another characteristic of the sample that distinguishes the two studies is the quantity and duration of the smoking habit. In the study performed by Sampaio et al. [22] all the individuals that comprised the sample had been smoking more than 20 cigarettes per day for over 15 years. In our study the smoking individuals that comprised the sample smoked between 10 and 20 cigarettes a day for a period that varied between 10 and 50 years.

This study suggests that the amount of cigarettes and the duration of the habit are not related with the variation of the number of AgNOR per nucleus. Zimmerman and Zimmerman [47] and Kapczinski [48] acknowledge the presence of cells alterations related to the number of cigarettes smoked a day and Silva [49] mentions the number of cigarettes smoked as a variable to be considered. Further studies are necessary for the evaluation of these findings when the silver soaking technique is applied for the evaluation of the AgNORs.

Studies evaluating the quantitative or qualitative differences of the AgNORs in smears collected from the oral mucosa according with the anatomic site were not found in the literature. In the present study there were no significant differences in the values obtained through the counting of the AgNORs in the non-smoking group when comparing the tongue border smears (1.79±0.21) and the floor of the mouth smears (1.84±0.23). This finding suggests there is no variation in these numbers concerning the anatomic site evaluated.

A larger number of AgNORs per nucleus was observed among the smoking individuals in the floor of the mouth smears (2.07±0.19) when compared to those collected from the tongue border (1.94±0.13), with a significant difference ($P = 0.0001$). These findings, plus the fact that these differences had not been observed in the non-smoking group, suggest that the non-keratinized epithelium of the oral mucosa is more susceptible to smoking than the other keratinized areas.

Corroborating the work of Sampaio et al. [22] in which was used the AgNOR silver soaking technique, and according to the cytology studies of Montgomery [50], Jacobs [51], Brown and Young [52] and Cowpe et al. [11], it was not found in this study correlations between gender, age and cellular alterations.

Smith and Crocker [53] bring out the possibilities of variation in AgNOR counting. In the present study an analysis of the percentage of nucleuses with more than

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three AgNORs was performed, what is believed to be an increase of the cellular proliferative activity within controlled patterns.

Through this analysis, there are significant differences ($P = 0.0001$) between the smokers group (25.16±6.01% on the tongue border and 30.51±8.25% on the floor of the mouth) and the non-smokers group (11.85±4.50% on the tongue border and 12.80±4.58% on the floor of the mouth) which suggest that the cigarette smoking habit is responsible for an increase in the cellular proliferative activity.

The average percentage of nuclei with more than three AgNORs analysis, when comparing the two anatomic sites, tongue border (25.16±6.01% in the smokers group and 11.85±4.50% in the non-smokers group) and floor of the mouth (30.51±8.25% in the smokers group and 12.80±4.58% in the non-smokers group) within the same group, showed a significant difference ($P = 0.0001$) for the smokers group, what did not happen for the non-smokers group. These findings are according to the earlier mentioned results, and corroborate the suggestion that the less keratinized oral cavity regions are more susceptible to the smoking action.

The analysis of the average percentage of nuclei with more than three AgNORs used as a parameter for a cellular proliferative activity evaluation is not frequent in the literature, being supported by the studies done by Giri et al. [42], where through the analysis of lung benign and malignant neoplasias the authors found a high number of nuclei with more than three AgNORs in the malignant neoplasias; and in the studies of Mesquita et al. [54], where the authors found a higher percentage of nuclei with more than three AgNORs in central ossifying fibromas than in the peripheral ones. Despite this parameter not being frequently used in studies, many authors [22,39,43,46,55] agree that there is graduate increasing in the AgNORs per nucleus distribution pattern, indicating a graduate increasing in cell proliferation.

The results obtained in this study suggest that the oral mucosa is susceptible to the cigarette smoking action, a susceptibility that is reflected by a cell proliferative activity increase. This tobacco effect on the cell proliferation is also related by Oijen et al. [56] and Sampaio et al. [22]. Even though we have presented significant differences, a noticeable number of overlapping was found in AgNORs counting among smokers and non-smokers patients, limiting this technique use as absolute criteria to close a final diagnosis on the cell differentiation stage. This information is coherent with the ones found by Vanheerden and Raubenheimer (1988). However, because it is a non-invasive and easily done procedure, the cytological AgNORs analysis may be useful as a long-term individual monitoring auxiliary method in oral cancer prevention for the risk group individuals. Prospective studies monitoring the average percentage and number of AgNORs and their relation with the developing of oral mucosal clinically visible alterations would be necessary to confirm this indication effectiveness.

According to Ogden et al. [34], there is a need to determine the sensibility, specificity and positive predictive value of this marker, to evaluate its use in a large population as well as its relation to other markers, since an unique marker present in every oral malignant neoplasias and absent from healthy oral mucosa has not being determined yet.

King [9] suggests that cytology, when used within a prevention program that includes an elaborate oral examination, other appropriate diagnosis methods and a patient monitoring, may become a sharp diagnosis adjunct method. The connection between the AgNORs evaluation made, dysplasias and malignant transformations remains unknown [39]. An interpretation of highly structured cell populations AgNORs counting, cells with high turnover as the healthy oral epithelium, may be a more complex process when comparing tissues of more stable cell populations; being the alteration process in a carcinoma dysplasia more complex than just a ploidy and cell proliferation alteration [44].

The meaning of the increase in oral cavity healthy tissue cell proliferation is also questioned by Sampaio et al. [22]. The present study suggests that there is an increase in the oral mucosal cell proliferation in smokers, being necessary more studies to define the meaning of this increase in malignant neoplasias and epithelium dysplasias development.

5. Conclusions

It is possible to conclude, according to the methodology used for this study and based on the results obtained, that:

1. The average number of AgNORs per nucleus found for individuals between 50 and 70 years old, filter cigarettes smokers, was of 1.94±0.13 in the tongue border and of 2.01±1.79 in the floor of the mouth. The average percentage of nucleuses with more than three AgNORs was of 25.16± 6.01% in the tongue border and of 30.51±8.25% in the floor of the mouth, in the smokers group.
2. There is a statistical difference ($P = 0.0001$) in the average number of AgNORs per nucleus and the average percentage of nucleuses with more than three AgNORs in individuals between 50 and 70 years old, filter cigarettes smokers and the averages found in the non-smokers group at the same age range, indicating a correlation between smoking habit and alterations in the oral epithelium cell proliferative activity.

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3. There is a statistical difference ($P = 0.0001$) in the average number of AgNORs per nucleus and the average percentage of nucleuses with more than three AgNORs in individuals between 50 and 70 years old, filter cigarettes smokers when comparing the two anatomic sites. There were no significant alterations in the two anatomic sites within the non-smokers group.
4. There is no correlation between age and gender to the quantity of AgNORs per nucleus in this sample.
5. It was not possible to correlate the smoking frequency to AgNORs variation in this sample.

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